PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/18, G01N 33/576 — A1

(11) International Publication Number: **WO 00/31130**

(43) International Publication Date: 2 June 2000 (02.06.00)

(21) International Application Number: PCT/IB99/01933

(22) International Filing Date: 19 November 1999 (19.11.99)

(30) Priority Data:
09/196,155 20 November 1998 (20.11.98) US

(71) Applicant: BIO MERIEUX [FR/FR]; Chemin de l'Orme, F–69280 Marcy l'Etoile (FR).

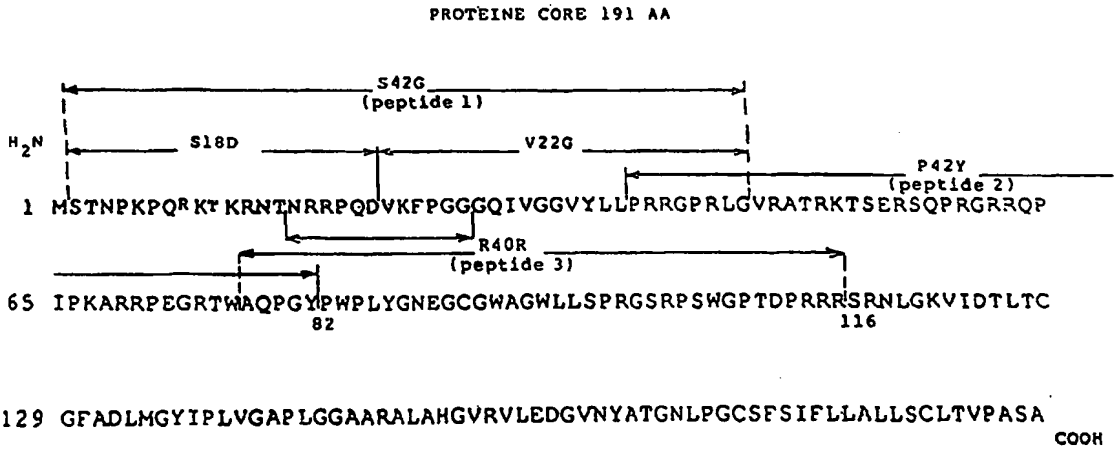
(72) Inventors: DALBON, Pascal; 6, boulevard Jules Favre, F–69006 Lyon (FR). JOLIVET, Michel; 16, avenue des Colonnes, F–69500 Bron (FR). JOLIVET–REYNAUD, Colette; 16, avenue des Colonnes, F–69500 Bron (FR).

(74) Agent: CABINET GERMAIN & MAUREAU; Boîte postale 6153, F–69466 Lyon Cedex 06 (FR).

(81) Designated States: CA, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: SYNTHETIC POLYPEPTIDES CORRESPONDING TO THE HEPATITIS C VIRUS (HCV) AND APPLICATIONS



(57) Abstract

Polypeptide that specifically binds to antibodies that specifically bind to human hepatitis C virus consisting of an antigenic sequence having specific immunoreactivity to the antibodies of SEQ ID NO:1, with the proviso that said polypeptide is different from SEQ ID NO:1, SEQ ID NO:2 and sequences resulting from the amputation of SEQ ID NO:1 with 1 to 10 amino acids from the N–terminal part and/or the C–terminal part of SEQ ID NO:1. Preferred polypeptides are selected from the group consisting of SEQ ID NO:8 through SEQ ID NO:18.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

SYNTHETIC POLYPEPTIDES CORRESPONDING TO THE HEPATITIS C VIRUS (HCV) AND APPLICATIONS

The present invention generally relates to synthetic polypeptides, that is to say which are obtained by preparative routes such as chemical synthesis, composed of consecutive amino acids which are together identical to any fragment, sequence or region of the structural protein of the nucleocapsid called CORE protein of the human hepatitis C virus (HCV). These polypeptides can be used as synthetic antigens in various applications arising from their immunogenicity and which are specified below; at the forefront of these applications is the detection HCV in various body fluids such as for example a blood sample.

It has been established that the nucleocapsid protein or CORE protein of HCV, which is composed as established by Figure 1 of 191 amino acids (SEQ ID NO:3), is that which exhibits the greatest homology, on the one hand, between the sequences of the same group of viral isolates, and, on the other hand, between the different groups of viruses. Moreover, this CORE protein is encoded by a structural part of the HCV genome and therefore constitutes a structural protein. The high conservation of the structure of this protein makes it a particularly suitable candidate for the immunological detection of HCV.

Thus, the work of Hosein B, Fang CT, Popovsky MA, Ye J, Zhang M, Wang CY, published in Improved sero-diagnosis of hepatitis C virus infection with synthetic peptide antigen from capsid protein, Proc Natl Acad Sci USA 1991; 88: 3647-51, made it possible to determine an immunodominant region in the CORE protein corresponding to a polypeptide consisting of the sequence of the N-terminal amino acids 1 to 120 of the said CORE protein.

In conformity with a publication already mentioned, namely Hosein B, Fang CT et al., Improved sero-diagnosis of hepatitis C virus infection with synthetic

peptide antigen from capsid protein, Proc Natl Acad Sci USA 1991; 88: 3647-51, various synthetic peptides corresponding to certain sequences of the CORE protein can be used as antigen in detection tests in a solid phase, for example on immunoabsorbent supports.

With the same objective of immunological detection of HCV, the document EP-0,442,394 describes several polypeptides comprising a polypeptide sequence belonging to the abovementioned immunodominant region of the CORE protein.

Among the said polypeptides, the one called VIIIE, consisting of the sequence of N-terminal amino acids 2 to 62 of the CORE protein, was tested in an ELISA test with respect to its immunoreactivity towards the anti-HCV antibodies contained in sera from individuals infected with HCV. This polypeptide demonstrated a good immunoreactivity towards the HCV-infected sera tested.

The substitution of such known polypeptides of the prior art, obtained by chemical synthesis, for the fusion protein corresponding to the CORE protein itself in tests of detection is advantageous since it makes it possible to reduce the risks of immunoreaction with antibodies which may be present in a sample and which are different from those directed against HCV.

However, it appeared essential to the Applicant to be able to determine a minimal and sufficient sequence for a polypeptide which, from the point of view of its antigenic properties, is equivalent to the protein in its entirety.

Indeed, the longer the peptide, the higher the risks capable of interfering with the antigenicity of the said peptide because of the higher frequency of the following events:

- interference between the peptide and antibodies different from those directed against HCV by cross-

reactions or between the peptide and other biological molecules present in the medium,

- conformational modifications relative to the structure of the native protein which may result in a disappearance of secondary and/or tertiary conformations corresponding to epitopic sites, or appearance of secondary and/or tertiary conformations different from those which the whole protein adopts, which are capable of interacting with antibodies other than the anti-HCV antibodies.

According to EP-0,442,394, the inventors have tried to shorten the length of the polypeptide VIIIE by respectively amputating 9, 19, 29 and 39 amino acids from its N-terminal part in order to prepare the polypeptides consisting of the N-terminal amino acid sequences of the CORE protein with a length of 10 to 62, 20 to 62, 30 to 62 and 40 to 62 respectively.

The immunoreactivity of each of these peptides was evaluated in ELISA tests and it is observed that the higher the number of amino acids amputated, the lower the immunoreactivity.

In contrast to these results, the present invention provides a polypeptide, or its fragments, which although consisting of an amino acid sequence much shorter than that of the VIIIE polypeptide structure manifests an immunoreactivity with all the sera of individuals or samples infected with HCV and which carry antibodies directed against the nucleocapsid protein.

The origin of the present invention is the following completely unexpected discoveries, which result from the experimental procedure outlined below:

1) an immunodominant region represented by at most the first 45 amino acids (SEQ ID NO:1) exists in the CORE protein of HCV;

2) this immunodominant region is sufficient by itself in order to obtain the same sensitivity as the total

CORE protein regarding the detection of anti-HCV antibodies;

3) this immunodominant region must be continuous if it is desired to react with all the sera of individuals or blood samples infected with HCV and which possess antibodies directed against the CORE protein;

4) this immunodominant region clearly contains conformational type epitopes and linear type epitopes.

Consequently, the polypeptide used in conformity with the invention comprises an isolated peptide sequence which is composed of about the 45 N-terminal amino acids of the HCV virus CORE protein (cf SEQ ID NO1).

Preferably, the polypeptide of the invention consists of only or of an isolated peptide sequence composed of the 45 N-terminal amino acids of the said protein or alternatively of any homologous polypeptide comprising about 45 amino acids and exhibiting an antigenic reactivity towards HCV.

Still preferably, the polypeptide of the invention consists of a peptide sequence which is composed of the N-terminal amino acids 2 to 45 of the CORE protein (cf SEQ ID NO2).

EP-A-0 569 309 relates to an isolated peptide sequence which is composed of 45 N-terminal amino acids (SEQ ID NO:1) of the CORE (or capsid) protein of the human hepatitis C virus (HCV), as represented in Figure 1, 1 to 10 amino acids being optionally amputated from this sequence in its N-terminal part and/or its C-terminal part.

"Isolated peptide sequence" is understood to mean any polypeptide not fused with another protein or another peptide regardless of its route of production, for example by chemical synthesis, by lysis of the CORE protein, or by genetic recombination techniques. This polypeptide can therefore be a synthetic peptide or a protein.

The preferred amputations of the polypeptide of the invention are the amputations of respectively 6 amino acids and 11 amino acids from the N-terminal end of the CORE protein.

The present invention concerns a polypeptide that specifically binds to antibodies that specifically bind to human hepatitis C virus consisting of an antigenic sequence having specific immunoreactivity to the antibodies of SEQ ID NO:1, with the proviso that said polypeptide is different from SEQ ID NO :1, SEQ ID NO :2 and sequences resulting from the amputation of SEQ ID NO :1 with 1 to 10 amino acids from the N-terminal part and/or the C-terminal part of SEQ ID NO :1.

The polypeptide sequences according to the invention may be such as in the native state or modified chemically. Chemical modification is understood to mean any chemical alteration of at least one functional group of the peptide sequence which essentially preserves or even develops the biological properties of the said sequence. The replacement of an amino acid of the L series by an amino acid of the D series, a modification of the side chains of the amino acids such as an acetylation of the amine functional groups, a carboxymethylation of the thiol functional groups or an esterification of the carboxylic functional groups, or a modification of the peptide bonds such as carba, retro-inverso, reduced and methylene-oxy bonds, are especially part of the chemical modifications considered above.

A preferred polypeptide (b) comprises or consists of an antigenic sequence which consists of SEQ ID NO:1 modified with at least one modification selected from the group consisting of :

(1) substituting an amino acid of SEQ ID NO:1 for at least one homologous amino acid, as defined below, and

(2) a chemical modification.

A preferred chemical modification is at least one member selected from the group consisting of :

(i) replacement of an amino acid of the L series by an amino acid of the D series,

(ii) modification of side chains of amino acids,

(iii) modification of peptide bonds.

Said modification of side chains may be an acetylation of amine functional groups, a carboxymethylation of thiol functional groups or an esterification of carboxylic functional groups.

Said modification of peptide bonds may be forming carba, retro-inverso, reduced and methylene-oxy bonds.

According to the present invention, an amino acid is said to be homologous to another amino acid when their chemical characteristics, such as polarity, hydrophobicity and/or basicity and/or acidity and/or neutrality, are essentially the same. In particular, a classification based on the polarity of the side chains plits up amino acids into four groups :

- non-polar or hydrophobic amino acids comprising :

  alanine, leucine, isoleucine, valine, proline,
  phenylalanine, tryptophane,
  methionine,

which are considered as homologous amino acids, within the meaning of the above definition,

- amino acids having a non-charged side chain comprising :

  serine, threonine, tyrosine,
  asparagine, glutamine,
  cysteine,
  glycine,

which are considered as homologous amino acids, within the meaning of the above definition,

- amino acids having a negative-charged side chain (acidic amino acids) comprising aspartic acid and glutamic

acid, which are considered as homologous amino acids, within the meaning of the above definition,

- amino acids having a positive-charged side chain (basic amino acids) comprising lysine, arginine, histidine, which are considered as homologous amino acids, within the meaning of the above definition ; depending on the pH, this group may further comprise asparagine and glutamine,

- rare amino acids comprising hydroxyproline, hydroxylysine, desmosine, isodesmosine, which are considered as homologous amino acids, within the meaning of the above definition.

According to another classification, asparagine and aspartic acid can be considered as homologous amino acids, and glutamine and glutamic acid, as well.

Another preferred polypeptide is shorter than SEQ ID NO:1, and shares at least one, preferably at least two identical amino acid with SEQ ID NO:5.

Preferebly, said polypeptide shares at least two identical amino acids with SEQ ID NO:6 and/or at least one identical amino acid with SEQ ID NO:7.

Said polypeptide may further have at least one amino acid which is homologous (as defined above) to at least one amino acid of SEQ ID NO: 1.

Said polypeptide may further have at least one amino acid which differs from at least one amino acid of SEQ ID NO:1 by a chemical modification (as defined above) of the side chain of said amino acid.

A preferred polypeptide is selected from the group consisting of SEQ ID NO:8 through SEQ ID NO:18.

The inventors have settled a screening test to determine whether a given polypeptide is an antigenic equivalent of polypeptide (a) or not. This test can be carried out by the one skilled in the art with routine experimentation and is described in details at the end of

the description (§ Antigenic polypeptide different from but equivalent to peptide (a)).

According to this test, the given polypeptide is screened against antibodies, at least one relevant antibody anti-HCV and one irrelevant antibody used as a negative control, using the usual methods to indicate the presence or absence of an antibody reaction. Examples of these methods include enzyme linked immunosorbent assay (ELISA). Suitable antibodies can either be purchased as commercially available antisera or prepared in a suitable host in accordance with well known procedures. The results can be expressed as the mean OD obtained with the relevant antibody - OD obtained with the irrelevant antibody. A threshold value is determined to select antigenic peptides.

In order to confirm this selection, the immunoreactivity of the selected peptides is tested with anti-HCV core positive human sera and with sera from healthy individuals. The results are expressed as the mean OD obtained with the pool of anti-core positive human sera - OD anti-core negative human sera.

From the above-defined peptide compounds or compositions, the invention provides a reagent for the detection of human hepatitis C virus (HCV) comprising as reactive substance any one of the abovementioned compounds or compositions and optionally any additive immunocompatible with the detection of HCV. Thus, the detection can be carried out using a polypeptide identical to those of the present invention or an antigenic equivalent peptide thereof, with optionally one anti-human immunoglobulin antibody, labelled with any conventional marker such as a radioactive, fluorescent or enzymatic marker or the like. Such a reagent can be used both in a homogenous phase, for example in immunoprecipitation assays, and in a heterogenous phase, for example in immunoadsorption assays.

With the abovementioned reagent, any suitable means of detection of HCV can be obtained, whether a detection kit or any other equivalent system or unit. By way of example, the abovementioned reagent is supported on a solid support immunocompatible with the reagent as a whole; in particular, the solid support is, without limitation, in the form of a microtiter plate, a sheet, a cone, a well, a bead or any other appropriate micro-particulate substrate.

The term solid support as used here includes all materials upon which the polypeptides according to the invention can be immobilized. These may be synthetic materials chemically modified or otherwise, especially polysaccharides such as cellulose materials, for example paper, cellulose derivatives such as nitrocellulose and cellulose acetate; polymers such as vinyl chloride, polyethylene, polystyrene, polyacrylate or copolymers such as vinyl chloride and propylene polymer, vinyl chloride and vinyl acetate polymer; styrene-based copolymers; natural fibers such as cotton and synthetic fibers such as nylon. Preferably, the solid support is a polystyrene polymer, a butadiene-styrene copolymer or a butadiene-styrene copolymer mixed with one or more polymers or copolymers chosen from polystyrene, styrene-acrylonitrile or styrene-methyl methylmethacrylate copolymers, polypropylenes, polycarbonates or analogs.

Using the immunological detection reagents or means according to the invention, anti-HCV antibodies can be detected in any body part or fluid such as a blood sample of an individual suspected of being infected with HCV. For that, this body part and the above-mentioned reagent simply have to be brought into contact under predetermined conditions, for example of temperature, which permit an immunological reaction where appropriate, and to then detect the presence of an immune complex with this reagent.

"Body part" is understood to mean any fluid, tissue or organ of an individual, comprising or capable of comprising anti-HCV antibodies. These body parts may be a blood, plasma or serum sample or various secretions and the like.

The process described above can be carried out in any detection device or apparatus comprising a vessel for bringing the body part analyzed into contact with a reagent as defined above, and this with means which create conditions, such as temperature, favorable for an immunological reaction where appropriate. And this device comprises means, especially optical, for the detection of the immune complex obtained with the reagent.

Another way of detecting the HCV virus using the polypeptides according to the present invention is to obtain monoclonal or polyclonal antibodies by any method known per se comprising an immunological reaction between a human or animal organism and an immunogenic agent consisting of a polypeptide composition as defined above. The antibodies thus obtained, for example conveniently labelled, can be used to detect HCV or to monitor the progression of the virus in a patient suffering from hepatitis C.

Of course, each of the polypeptide compositions according to the invention may constitute the active ingredient of an active immunotherapeutic composition, being optionally conjugated with an immunologically suitable support. A pharmaceutically acceptable excipient may supplement the said composition. Such a composition is for example a vaccinal preparation.

The immunodominant character of the peptide sequence according to the present invention was demonstrated in conformity with the following experimental procedure.

The strategy chosen consists in synthesizing long polypeptide fragments of about 40 amino acids, in the

N-terminal part of the CORE protein, which belong to the sequence of about the first 120 amino acids.

In a first stage, three peptides were therefore defined, beginning the synthesis at amino acid No. 2 (serine).

In conformity with Figure 1, three peptides were synthesized, namely:

- peptide called S42G, extending from serine 2 up to glycine 45
- peptide called P42Y, extending from proline 39 up to tyrosine 82
- peptide called R40R, extending from arginine 75 up to arginine 116.

It appears that these peptides have some amino acids in common, which makes it possible to identify a possible antigenic determinant located at the intersection of two peptides.

The peptides were chemically synthesized by solid phase synthesis according to the Merrifield technique (Barany G, and Merrifield R.B, 1980, In the Peptides, 2, 1-284, Gross E and Meienhofer J, Eds Academic Press, New York). The practical details are those described below.

Peptide synthesis

The peptides are synthesized on a phenyl-acetamidomethyl (PAM)/polystyrene/divinylbenzene resin (Applied Biosystems, Inc. Foster City, CA), using an automatic "Applied Biosystems 430A" synthesizer. The amino acids are coupled in the form of esters of hydroxy-benzotriazole (HOBT). The amino acids used are obtained from Novabiochem (Laüflerlfingen, Switzerland) or from BACHEM (Bubendorf, Switzerland).

Chemical synthesis of the peptides was carried out using a double coupling procedure with N-methylpyrrolidone (NMP) as solvent. The peptides were simultaneously cut from their resin as well as the side protections using

hydrofluoric acid (HF) in a suitable apparatus (type I cutting apparatus, Peptide Institute, Osaka, Japan).

For 1 g of peptidylresin, 10 ml of HF, 1 ml of anisole and 1 ml of dimethyl sulfide (DMS) are used, and the mixture is stirred for 45 minutes at -2°C. The HF is then evaporated under vacuum. After intensive washes with ether, the peptide is eluted from the resin with 10% acetic acid and then the peptide is freeze-dried.

The peptides are purified by preparative high-performance liquid chromatography on a type C18 VYDAC column (250 x 21 mm) (The Separation Group, Hesperia, CA, USA). The elution is performed with an acetonitrile gradient at a flow rate of 22 ml/min. The fractions collected are controlled by elution under isocratic conditions on an analytical C18 VYDAC column (250 x 4.6 mm) at a flow rate of 1 ml/min. The fractions which have the same retention time are pooled and freeze-dried. The predominant fraction is then analyzed by analytical high-performance liquid chromatography with the system described above. The peptide which is considered as being of acceptable purity results in a single peak representing 95% of the chromatogram minimum.

The purified peptides are analyzed with the objective of assessing their amino acid composition using an automatic Applied Biosystems 420 H amino acid analyzer. Measurement of the chemical molecular mass (mean) of the peptides is obtained using the L.S.I.M.S. mass spectrometer in a positive ion mode, on a dual focusing instrument VG. ZAB.ZSEQ linked to a DEC-VAX 2000 acquisition system (VG analytical Ltd, Manchester, England).

The reactivity of these three peptides towards the sera of individuals infected with the hepatitis C virus, termed (HCV) positive, was evaluated in an ELISA test according to the procedure described below.

Detection of anti-HCV antibodies by ELISA

The wells of a microtiter plate of "NUNC maxisorb" trademark are saturated with 100 µl of a solution containing the peptide or a mixture of peptides (10 µg/ml) for 2 hours at 37°C. The plate is then emptied, then washed with a wash buffer containing 0.05% Tween 20. The wells are saturated with 100 µl of wash buffer supplemented with 10% goat serum (v/v), then incubated for 30 minutes at 37°C, then washed again as above. The sera to be analyzed are diluted to the appropriate dilution with saturation buffer. The incubation of the sera is 1 hour at 37°C. The wells are again washed. The solution of conjugate (goat IgG to human IgG labelled with peroxidase) at a dilution of 1/1000 in the saturation buffer is then added and the incubation lasts for 90 minutes at 37°C. After washing, the solution of ortho-phenylenediamine substrate is added. After 10 minutes, the reaction is stopped with 50 µl of H_2SO_4 and the optical density is read at 492 nm. It should be noted that all the tests were carried out in duplicate.

The reactivity of the peptides S42G, P42Y, and R40R is measured by ELISA on HCV-positive sera (P 1 to P 20 and B 1 to B 16) and on normal sera (SN 10, 11, 16, 17, 18, 19).

For that, the different peptides are adsorbed on the microplates at a concentration of 10 µg/ml and the sera are used at 1/100 dilution.

The values obtained, which are collated in Table 1 below, correspond to the optical density (OD) multiplied by 10^3, at 492 nm.

For each serum the experiment was carried out in duplicate. The **** are values outside the upper scale.

TABLE 1

	S42G	R42Y	R40R		S42G	P42Y	R40R
P 1	*****	101	375	B 1	*****	869	172
	*****	108	420		*****	811	173
P 2	*****	119	399	B 2	2302	1749	364
	*****	104	391		2276	1664	345
P 3	2145	648	223	B 3	1673	623	304
	1942	638	215		1686	630	341
P 4	*****	2314	309	B 4	*****	1688	405
	*****	2105	307		*****	1557	346
P 5	*****	234	129	B 5	*****	1639	360
	*****	243	176		2308	1699	281
P 6	116	184	87	B 6	1671	810	172
	130	185	100		1778	791	163
P 7	*****	2295	496	B 7	*****	1657	418
	*****	2389	478		*****	1489	457
P 8	*****	983	282	B 8	1567	611	271
	*****	903	328		1543	620	259
P 9	186	238	159	B 9	*****	957	235
	183	231	158		*****	913	229
P 10	169	194	218	B 10	360	227	108
	177	204	216		386	223	98
P 11	*****	*****	1191	B 11	1749	813	164
	*****	*****	1377		1849	789	184
P 12	*****	*****	1121	B 12	*****	755	136
	*****	*****	1231		*****	407	117
P 13	114	64	113	B 13	1341	746	140
	106	108	116		1142	609	99
P 14	*****	362	280	B 14	455	246	130
	*****	349	270		450	259	125
P 15	*****	*****	2305	B 15	*****	313	301
	*****	*****	2335		*****	312	303
P 16	*****	1742	938	B 16	*****	222	117
	*****	1667	964		*****	153	125
P 17	*****	799	217	SN 10	205	237	163
	*****	736	212		192	205	154
P 18	*****	2253	1427	SN 11	107	156	150
	*****	2339	1327		100	141	138
P 19	105	106	84	SN 16	551	657	426
	112	105	89		537	667	439
P 20	*****	1701	714	SN 17	129	156	104
	*****	1679	740		122	144	74
				SN 18	218	332	119
					173	279	87
					139	167	480
				SN 19	120	161	496

Table 1 shows that the peptides react differently with the sera.

It appears clearly that the most reactive peptide is peptide S42G which detects 31 sera out of 36.

None of these peptides detects normal sera, which confirms their specificity.

Finally, no serum which is negative with peptide S42G is positive with peptides P42Y or R40R, which shows that on its own, peptide S42G detects the sera without the help of the other two peptides.

The study was then continued in order to know more precisely the antigenic determinant(s) located on peptide S42G.

For this purpose, two peptides were prepared under the same conditions as above.

These two peptides are, in conformity with Figure 1:

1) a peptide of 20 amino acids, called S18D, covering sequence 2 to 21 of the CORE protein
2) a peptide of 24 amino acids, called V22G, covering sequence 22 to 45 of the CORE protein.

The reactivity of these two peptides (separated and combined) was evaluated by comparing it with that of peptide S42G in an ELISA test as described above.

The reactivity of peptides S42G, S18D, V22G, S18D + V22G is measured by ELISA on HCV-positive sera. The different peptides are absorbed onto microplates at a concentration of 10 µg/ml and the sera are used at the dilution stated.

The values obtained, which are collated in Table 2 below, correspond to the optical density at 492 nm. All the experiments were carried out in duplicate.

TABLE 2

	SERUM		S42G	S18D-V22G	S18D	V22G
1	P1	1/100	2.500	0.438	0.012	0.426
2	P2	1/100	2.500	0.310	0.020	0.290
3	P3	1/100	2.500	0.665	0.162	0.503
4	P4	1/100	2.500	2.500	0.982	1.739
5	P5	1/100	2.500	2.500	2.500	2.500
6		1/1000	2.500	2.500	2.317	2.293
7		1/10000	2.093	0.977	0.399	0.578
8	P6	1/100	0.000	0.007	0.007	0.000
9	P7	1/100	2.500	2.500	2.500	2.500
10		1/1000	2.500	2.500	0.854	1.590
11		1/10000	1.916	0.402	0.165	0.237
12	P8	1/100	2.500	2.500	2.500	2.241
13		1/1000	2.500	2.500	2.500	2.476
14		1/10000	1.565	0.730	0.681	0.049
15	P9	1/100	0.090	0.027	0.019	0.008
16	P10	1/100	0.172	0.054	0.028	0.026
17	P11	1/100	2.500	2.500	0.383	2.500
18	P12	1/100	2.500	2.500	2.500	2.500
19		1/1000	2.500	2.500	2.500	2.500
20		1/10000	2.500	0.560	0.454	0.106
21	P13	1/100	0.000	0.025	0.012	0.013
22	P14	1/100	2.500	2.500	0.907	1.778
23	P15	1/100	2.500	2.500	2.500	1.810
24	P16	1/100	2.500	2.500	0.225	2.500
25	P17	1/100	2.500	2.129	0.297	1.832
26	P18	1/100	2.500	2.500	2.500	2.500
27		1/1000	2.500	0.895	0.297	0.598
28		1/10000	1.006	0.167	0.095	0.072
29	P19	1/100	0.000	0.021	0.011	0.000
30	P20	1/100	2.500	2.500	1.433	2.249
31	P21	1/100	2.500	2.383	0.111	2.272
32	P22	1/100	2.500	2.500	2.500	2.500
33		1/1000	2.500	1.844	1.142	0.702
34		1/10000	0.894	0.234	0.146	0.088
35	P23	1/100	0.000	0.030	0.015	0.015
36	P24	1/100	2.500	0.594	0.015	0.579
37	P25	1/100	2.500	2.500	2.500	2.252
38		1/1000	2.500	2.500	2.199	0.695
39		1/10000	1.550	0.418	0.329	0.089
40	P26	1/100	2.500	2.500	2.500	2.500
41		1/1000	2.500	2.500	2.500	1.541
42	P26	1/10000	2.500	1.156	0.957	0.199
43	P27	1/100	2.500	2.500	1.425	1.600
44	P28	1/100	2.500	2.500	0.115	2.500
45	P29	1/100	0.331	0.905	0.000	0.005
46	P30	1/100	2.500	2.500	0.483	2.500
47	P31	1/100	2.500	2.500	2.500	2.500
48		1/1000	2.500	2.500	1.975	2.500
49		1/10000	2.071	1.030	0.183	0.847
50	P32	1/100	2.500	2.500	1.046	1.639
51	P33	1/100	2.500	2.500	1.307	1.987
52	P34	1/100	2.500	2.500	2.500	1.618
53	P35	1/100	2.500	2.500	2.500	1.504
54	P36	1/100	2.500	1.341	0.115	1.228
55	P37	1/100	2.500	2.500	2.500	2.500
56		1/1000	2.500	1.388	0.523	0.865
57		1/10000	1.088	0.230	0.102	0.128
58	P38	1/100	2.500	2.500	2.500	2.500
59		1/1000	2.500	2.335	0.753	1.582
60		1/10000	1.477	0.156	0.099	0.057
61	P39	1/100	2.500	2.500	2.500	1.835
62	P40	1/100	2.500	2.500	2.500	2.500
63	P41	1/100	2.500	2.500	1.579	2.218

64	P42	1/100	0.034	0.000	0.000	0.000
65	CTS	1/100	2.500	2.500	2.500	2.500
66		1/1000	2.500	2.500	2.500	1.155
67		1/10000	2.500	0.338	0.295	0.043
68		1/100	2.500	2.500	2.500	2.500
69		1/1000	2.500	2.500	2.500	1.605
70		1/10000	1.336	0.602	0.347	0.255
71	B1	1/100	2.500	2.500	1.859	1.225
72	B2	1/100	2.500	2.500	1.781	0.756
73	B3	1/100	2.500	1.573	1.244	0.329
74	B4	1/100	2.500	2.500	2.500	2.500
75		1/1000	2.500	2.464	1.250	1.214
76		1/1000	1.021	0.315	0.171	0.144
77	B5	1/100	2.500	2.500	2.032	0.863
78	B6	1/100	2.500	2.500	2.500	0.749
79	B7	1/100	2.500	2.500	2.500	2.102
80	B8	1/100	2.500	1.720	1.362	0.358
81	B9	1/100	2.500	2.500	0.808	2.082
82	B10	1/100	0.721	0.324	0.099	0.225
83	B11	1/100	2.084	2.500	2.324	0.616
84	B12	1/100	2.500	2.392	1.375	1.017
85	B13	1/100	1.809	0.674	0.370	0.304
86	B14	1/100	0.698	0.258	0.072	0.186
87	B15	1/100	2.500	1.044	0.090	0.954
88	B16	1/100	2.500	2.500	2.500	2.500

Each serum was, in a first instance, tested at the dilution 1/100. In the event where the response proved saturating (value 2500) for all the peptides (example: serum P 5) a 1/1000 dilution, and if necessary a 1/10,000 dilution, was carried out.

It appears that for all the HCV-positive sera, the reactivity of peptide S42G is substantially greater than the reactivity of peptides S18D and V22G, and than that of S18D + V22G.

The sera P6, P9, P10, P13, P19, P23, P24 are sera which do not possess antibodies against the CORE protein of HCV.

Although these results as a whole are unambiguous, the attachment of the different peptides to the wells of the microtiter plates can modify the epitopes or determinants of the peptide tested. The plates used (NUNC

Maxisorb) are polystyrene plates irradiated with gamma rays, which bind the peptides in a noncovalent manner via electrostatic type bonds but also hydrophobic bonds. It is possible that peptides, depending on their sequence, are selectively adsorbed, thus favoring a well defined part and thus preventing immunogenic reactivity towards another part which may have become less acceptable.

To evaluate this hypothesis, inhibition tests, whose usefulness lies in the fact that they allow the formation of the antigen-antibody complex in liquid medium, were carried out, thus dispensing with possible artifacts linked to the adsorption of peptides onto a solid support.

The methodology is that described below.

Inhibition test

The inhibition experiments were carried out by reaction, in liquid phase, of the HCV sera with the peptides followed by reaction of the remaining antibodies with the peptide adsorbed onto the microplates. The inhibitory peptides are incubated at a concentration of 0.1 mg/ml with sera of appropriate dilution. The rest of the manipulation is identical to that described for the ELISA test.

Peptide S18D, or V22G, or a mixture of both, is preincubated overnight in the presence of the serum to be tested. The antibodies can bind onto the corresponding sites. The mixture (peptide + serum) is then incubated with the peptide S42G adsorbed onto the microtiter plates. If all the antibodies reacted during the incubation with the peptides S18D, V22G, or with the mixture, no reactivity will be observed, which will result in a 100% inhibition. In contrast, if antibodies specific for peptide S42G remain, they will then be able to react.

A control is carried out by preincubating each serum with peptide S42G, which makes it possible to calculate the percentage inhibition.

Table 3 collates the results of the inhibition of the binding of anti-HCV antibodies (dilution 1/10,000) onto peptide S42G, by preincubation of HCV sera with peptide S42G, peptide S18D, peptide S22G and the mixture of peptides S18D + S22G.

TABLE 3

	Serum	Inhibition S42G	Inhibition S18D	Inhibition V22G	Inhibition pool S18D + V22G
1	P5	100%	3.5%	83.0%	77.0%
2	P7	100%	8.4%	81.0%	86.0%
3	P8	100%	1.4%	77.4%	53.0%
4	P18	100%	10.6%	48.0%	52.0%
5	P22	100%	5.6%	65.5%	57.0%
6	P25	100%	14.1%	71.8%	70.0%
7	P31	100%	2.0%	39.9%	27.0%
8	P37	100%	43.6%	88.7%	73.0%
9	P38	100%	8.9%	70.3%	65.0%
10	B4	100%	16.8%	83.6%	72.0%

As shown in Table 3, no peptide completely inhibits the reactivity of the sera towards peptide S42G.

In other words, this experiment proves that antibodies specific for peptide S42G exist which do not react with either peptide S18D or with peptide V22G, the sum of both representing the total sequence of peptide S42G.

A final hypothesis to be evaluated consists in verifying that the antibodies specific for peptide S42G were not directed against the central part of peptide S42G, that is to say at the junction of peptides S18D and V22G.

A peptide was therefore prepared (cf Figure 1) whose sequence comprises the C-terminal part (6 amino acids) of peptide S18D and the N-terminal part (6 amino acids) of peptide V22G.

Although this peptide exhibits reactivity with HCV-positive sera, the level obtained is in no case comparable to that obtained with peptide S42G.

The set of results presented above makes it possible to draw the following conclusions.

In the 120 N-terminal amino acids of the CORE protein, and more particularly in the first 62 amino acids, the first 45 amino acids are the most reactive towards HCV-positive sera.

The first 21 amino acids (peptide S18D) react, which shows the presence of one or more antigenic determinants on this peptide.

The amino acids 22 to 45 (peptide V22G) also carry one or more epitopes.

The junction of these two sequences is also reactive.

Consequently, the sequence 1-45 of the CORE protein is pluriepitopic.

Furthermore, one or more antigenic determinants exist which are reactive only insofar as the entire sequence 2-45 is available and not in a discontinuous manner (peptides S18D + V22G). These epitopes, which are specific to peptide S42G, are without any doubt conformational type epitopes which can exist only insofar as this sequence of 44 amino acids (peptide S42G) has a suitable structure, a structure which is not obtained with smaller-sized peptides.

If the amino acid sequence of peptide S42G should not be discontinuous in order to preserve all the epitopes, it can be asked if the N- and/or C-terminal parts of peptide S42G are involved in the epitopic conformations of S42G or carry the epitopes themselves.

In order to try to respond, five peptide fragments derived from S42G by N- and/or C-terminal amputations were defined.

In conformity with Figure 2, the following five fragments were synthesized according to the Merrifield technique in conformity with the procedure described above:

- peptide called P37G corresponding to the amino acid sequence 7 to 45 of the CORE protein and to an

amputation of 5 amino acids from the N-terminal part of S42G,

- peptide called K32G corresponding to the amino acid sequence 12 to 45 of the CORE protein and to an amputation of 10 amino acids from the N-terminal part of S42G,
- peptide called S32Y corresponding to the amino acid sequence 2 to 35 of the CORE protein and to an amputation of 10 amino acids from the C-terminal part of S42G,
- peptide called P27Y corresponding to the amino acid sequence 7 to 35 of the CORE protein and to an amputation of 5 amino acids from the N-terminal part and of 10 amino acids from the C-terminal part of S42G,
- peptide called K22Y corresponding to the amino acid sequence 12 to 35 of the CORE protein and to an amputation of 10 amino acids from the N-terminal part and of 10 amino acids from the C-terminal part of S42G.

The reactivity of the five peptides towards sera of individuals infected with HCV was evaluated in ELISA tests in conformity with the procedure described above for measuring the activity of the peptides S42G, P42Y and R40R.

Table 4 below collates the results obtained for the peptides S42G, S32Y, P27Y, K22Y in order to examine the influence of an amputation of the N-terminal part and the C-terminal part of the peptide S42G.

These results are expressed in optical density values read at 492 nm multiplied by a factor of 10^3.

TABLE 4

Serum	Dilution	RIBA C22	S42G	S32Y	P27Y	K22Y
P 1	1/100		D	703	620	435
P 2	1/100		D	1177	891	666
P 3	1/100		D	1576	1470	1031
P 23	1/100		146	157	138	125
P 10	1/100		56	78	60	66
P 17	1/100		D	D	2300	1930
P 24	1/100		D	1214	1063	735
P 29	1/100		594	400	347	310
P 30	1/100		D	D	2274	1805
P 32	1/100		D	D	D	D
B 3	1/100		D	D	D	2033
P 4	1/100		D	D	D	D
	1/1000		D	2165	2111	1959
	1/10,000		588	369	324	300
P 5	1/100		D	D	D	D
	1/1000		D	D	D	D
	1/10,000		1880	1242	856	394
P 7	1/100		D	D	D	D
	1/1000		D	D	D	D
	1/10,000		2024	1932	1667	1487
P 8	1/100		D	D	D	D
	1/1000		D	D	D	2257
	1/10,000		1423	1068	654	335
P 14	1/100		D	D	D	D
	1/1000		D	D	D	D
	1/10,000		1296	601	566	398
P 16	1/100		D	D	D	D
	1/1000		D	983	913	610
	1/10,000		750	124	104	75
A 8	1/100	4	D	D	D	D
	1/1000		D	1372	1198	431
	1/10,000		620	173	118	56
A 9	1/100	4	D	D	D	2152
	1/1000		D	1941	1388	282
	1/10,000		394	297	182	43
A 10	1/100	4	D	D	2308	2114
	1/1000		D	373	334	207
	1/10,000		819	33	31	26
A 11	1/100	4	D	2240	1937	1878
	1/1000		1865	228	226	212
	1/10,000		223	36	33	31
A 12	1/100	4	D	D	D	D
	1/1000		D	D	D	646
	1/10,000		848	588	365	62

The sera P23 and P10 are sera which do not possess antibodies against the CORE protein of HCV.

From this Table, it can be deduced that when the

10 amino acids of its C-terminal part are amputated, S42G loses its reactivity and that furthermore if 5 and 10 amino acids are respectively amputated from its N-terminal part, this results in a reduction in immunoreactivity which increases as a function of decreasing peptide length. (cf Fig. 2, and in particular the sera P2, P5 and P8 of Table 4).

Table 5 collates the results of the tests of immunoreactivity of the peptides S42G, P37G, K32G, during ELISA tests, to examine the influence of amputation of the N-terminal part of peptide S42G.

The values given correspond to the optical density read at 492 nm multiplied by the factor 10^3.

TABLE 5

Serum	Dilution	S42G	P37G	K32G
A 8	1/1000	D	D	D
	1/10,000	921	525	666
A 9	1/1000	D	2059	1675
	1/10,000	672	275	229
A10	1/10,000	1485	1215	
A11	1/1000	D	D	2108
	1/10,000	397	321	343
A12	1/1000	D	D	D
	1/10,000	1418	823	598
A13	1/1000	D	D	D
	1/10,000	1519	1061	1247
A14	1/1000	1407	539	998
	1/10,000	149	65	103
A15	1/1000	D	D	D
	1/10,000	1357	905	715
A16	1/1000	D	D	D
	1/10,000	D	2003	D
A19	1/1000	D	D	D
	1/10,000	620	446	594
A20	1/1000	D	D	D
	1/10,000	D	2338	D
A21	1/1000	1319	652	993
	1/10,000	177	90	123
A22	1/1000	1216	702	876
	1/10,000	164	102	129
A23	1/1000	D	D	D
	1/10,000	860	557	774

These results strengthen us regarding the hypothesis according to which peptide S42G must be present in its entire sequence from 2 to 45 in order to exhibit maximum immunoreactivity.

In all cases, S42G is higher than P37G, which indicates that the 5 N-terminal amino acids play a role in the antigenicity.

However, in certain cases, little or no difference is observed in reactivity between peptides P37G and K32G, which would tend to prove that amino acids 7 to 11 are not of major importance for the antigenicity of peptide S42G.

Moreover, a comparison of Tables 4 and 5 makes it possible to demonstrate the importance of the 10 C-terminal amino acids of peptide S42G in the immuno-reactivity itself.

Finally, the entire CORE protein (191 amino acids) was replaced by peptide S42G (44 amino acids), to detect the anti-HCV antibodies.

For this, the choice was made to compare the sensitivity of peptide S42G to that of the 2nd generation ORTHO HCV ELISA test; it is a test marketed by the company ORTHO which comprises a fusion protein incorporating the CORE protein of HCV, called C22-3; cf Vanderpoel, C.L., HTM Cuypers, H.W Reesink et al., 1991, Confirmation of hepatitis C virus infection by new four antigen recombinant immunoblot assay, Lancet 337; 317-319.

The comparison was carried out on 173 samples which were positive with the 2nd generation ORTHO HCV ELISA test.

Of 173 samples, the peptide S42G detected 151 of them, which gives a sensitivity of 87.28%. The 22 discordant sera were then analyzed using another 2nd generation test, namely CHIRON RIBA HCV. It is an immunoblotting intended for the detection of antibodies directed against the hepatitis C virus antigens in human serum or plasma. This test comprises five recombinant antigens (proteins).

One of them is the recombinant CORE protein C22-3 obtained in the form of a fusion protein with human superoxide dismutase and expressed by a yeast.

It is found at the end of this confirmatory test that none of the 22 sera exhibits reactivity towards the band C22-3 (CORE).

Consequently, the sensitivity of peptide S42G is 100% relative to the CORE protein (C22-3) of the 2nd generation CHIRON RIBA HCV test.

In conclusion, the CORE protein can be replaced by the peptide S42G for the serological detection of HCV.

At this stage of the description of the invention, it is appropriate to demonstrate the advantageous use of synthetic peptides relative to that of recombinant protein fragments. For that, the results and experimental observations according to the invention were compared with those of the publication, namely: Nasoff MS, Zebedee SL, Inchauspe G, Prince AM, Identification of an immunodominant epitope within the capsid protein of hepatitis C virus, Proc Natl Acad Sci USA 1991; 88: 4641-5. This publication relates to the production of recombinant protein fragments of the CORE protein expressed in E. coli, in conformity with Figure 3, and reports results which are both similar to and different from those reported above.

Indeed, the authors have expressed a recombinant protein comprising the first 74 amino acids of the CORE protein. The cloning strategy used results in the production of fusion proteins. In other words, the 74 N-terminal amino acids of the HCV CORE protein called CAP-A are preceded by 308 amino acids of which the first 221 correspond to glutathione S-transferase. The reactivity of this protein of 382 amino acids, of which only 20% represent the CORE protein, towards HCV-positive sera is good only in appearance given the very small number of sera tested (5 human sera).

In contrast, a protein comprising the sequence 69-120 of the CORE protein, called CAP-B, exhibits no reactivity towards these same sera. This last result is in relative contradiction with those of the present invention since the peptide R40R which comprises the sequence 75-116 of this same protein reacts nevertheless with some sera (about 10%, cf Table 1).

These same authors pursued their work, producing other recombinant fusion proteins with, as sequence of the CORE protein, the amino acids 1-20, 21-40, 41-60 respectively, called CAP-1, CAP-2 and CAP-3 respectively.

Their results, still obtained on a very small number of human sera (9 sera), show that the sequence 21-40 reacts better than the sequence 1-20. The sequence 41-60 exhibits for its part no reactivity. This last result is also in relative contradiction with those presented according to the invention since the peptide P42Y (amino acids 39-82) exhibits (cf Table 1) a high reactivity although less than the peptide S42G.

Furthermore, another publication, namely: Okamoto H, Munekata E, Tsuda F, Takahashi K, Yotsumoto C, et al., 1990, Jpn, J. Exp Med 60, 223-233, has shown that a peptide of 36 amino acids comprising the sequence 39-74 of the CORE protein reacts with at least 70% of HCV-positive sera.

Regarding the reactivity of the sequence 21-40, these authors assert that in several cases, the reactivity of this recombinant CORE protein fragment is greater than that comprising the sequence 1-74.

The results obtained according to the invention are not in agreement with the results of these authors since it has been demonstrated that in all the cases of HCV sera studied (cf Table 2), the reactivity of the peptide S42G (sequence 2-45) is substantially greater than that of the peptides S18D (sequence 2-21) and V22G (sequence 22-45).

The explanation proposed to explain these divergent results relates to the production of different fragments of the CORE protein in both cases.

According to the invention, the peptides obtained by chemical synthesis comprise only the sequence mentioned for each of them, and as explained above, this is one of the advantages linked to these synthetic peptides.

In the case of the recombinant proteins obtained by NASOFF et al., they are fusion proteins in which 308 amino acids, which are completely foreign to the CORE protein, are present in the N-terminal position.

The recombinant proteins which comprise the sequences 1-20 and 21-40 of the CORE protein are therefore composed of a foreign sequence by more than 90%.

Although the authors stipulate that for the detection tests, the glutathione S-transferase part of the fusion protein does not disrupt the reaction since none of the sera tested reacts with isolated glutathione S-transferase (therefore no false positives), it appears difficult to admit that these 90% of fusion protein do not in any way interfere in the reactivity with the anti-HCV antibodies.

Indeed, the fact that the N-terminal part of the sequences 1-20 or 21-40 of the CORE protein is linked to the C-terminal part of the fusion protein contributes towards restricting the accessibility of this N-terminal region. In contrast, the C-terminal part is for its part detected. It is highly probable that it is these structural stresses, imposed by the production of a recombinant fusion protein in which the immunogenic part (the CORE part) represents the minor part of the fusion protein (less than 10% in this case), which lead to results contrary to those presented.

The amino acids are represented according to Figures 1 and 2, according to the convention of the Table below:

TABLE 6

AMINO ACID	3 LETTER CODE	MOLECULAR WEIGHT
ALANINE	Ala	S9
CYSTEINE	Cys	121
ASPARTIC AC.	Asp	133
GLUTAMIC AC.	Glu	147
PHENYLALANINE	Phe	165
GLYCINE	Gly	75
HISTIDINE	His	155
ISOLEUCINE	Ile	131
LYSINE	Lys	146
LEUCINE	Leu	131
METHIONINE	Met	149
ASPARAGINE	Asn	132
PROLINE	Pro	115
GLUTAMINE	Gln	146
ARGININE	Arg	174
SERINE	Ser	105
THREONINE	Thr	119
VALINE	Val	117
TRYPTOPHAN	Trp	204
TYROSINE	Tyr	181

According to the invention, the complete experimental procedure described above clearly demonstrates, by the types of antigen-antibody reaction carried out either in solid phase (direct ELISA, cf Table 2), or by inhibition (cf Table 3), that the sequence 2-45 of the CORE protein of the HCV virus obtained by solid phase chemical synthesis not only proves substantially greater than smaller sequences (S18D or V22G), but also that it exhibits a sensitivity equivalent to the CORE protein itself (protein C22-3 of the 2nd generation CHIRON RIBA HCV test), and that consequently the synthetic peptide S42G can be used in serological diagnostic tests in place of the CORE protein.

It is evident from all these results that peptide S42G appears to be the minimum but sufficient structure which, from the point of view of its antigenic properties, is equivalent to the CORE protein in its entirety and can therefore replace it in a reagent for the detection of

HCV.

Antigenic polypeptide different from but equivalent to peptide (a),

Structural analysis and molecular modeling of the peptide S42G allowed the characterization of a tridimensional motif that is composed of 2 a-helix separated by a loop. This structure could define a conformation-dependent antigenic domain. As an attempt to obtain mimotopes which could mimic this domain, we have screened a dodecapeptide library displayed on phage (Ph.D.-12TM Phage Display Peptide Library Kit, New England BioLabs Inc) with the mouse monoclonal antibody (Mab) 19D9D6. This Mab was directed against a conformational epitope present within this region and also recognized by human sera.

Four rounds of biopannings with decreasing amounts of Mab 19D9D6 were performed according to the instructions of the manufacturer manual. Then 72 clones were randomly selected and their inserts were sequenced. Twenty three different deduced aminoacid sequences were obtained. However, among these different sequences, 5 were represented 35, 10, 4, 3 and 2 times respectively. The different peptides displayed on phage were tested for target binding by ELISA with either Mab 19D9D6 (anti-HCV) or an irrelevant Mab (anti OSPA Mab) used as a negative control. Eleven mimotopes were specifically recognized by Mab 19D9D6 and not by the anti OSPA Mab. However, 1 out of the 11 mimotopes, the sequence of which was overrepresented (WPHNWWPHFKVK), appeared to be highly immunoreactive compared to the other peptides. Interestingly, homology search using the clustalw aligment (Mac Vector software 6.0.1, Oxford Molecular group PLC) indicated that this mimotope did not share any obvious homology with the core region previously defined by spotscan as critical for MAb 19D9D6 binding whereas only a

few homologies with aminoacids contained within this region were found with other mimotopes (figure 4). They allowed to localize the MAb 19D9D6 epitope on the tridimensional structure of S42G.

Table 7

N° of clone	Clone sequence	Nb of clones /72	OD MAb 19D9D6-OD antiOSPA MAb
1	WPHNWWPHFKVK SEQ ID NO:8	35	1.956
2	HLNILSTLWKYR SEQ ID NO:9	1	1.114
3	HKHAHNYRLPFS SEQ ID NO:10	4	0.714
4	PKVLIVVLEASG SEQ ID NO:11	1	0.672
5	QVYAEFKTSFRS SEQ ID NO:12	1	0.592
6	GHIHSMRHHRPT SEQ ID NO:13	10	0.559
7	TSFHSKAEYTGI SEQ ID NO:14	1	0.559
8	KDVDTSQDKMYW SEQ ID NO:15	1	0.507
9	QNSSMMLVPWRT SEQ ID NO:16	1	0.481
10	AETVESCLAKSH SEQ ID NO:17	1	0.456
11	SLRLGIVLLWKL SEQ ID NO:18	1	0.444

The immunoreactivity of phage clones was tested according to the manufacturer manual. The results were expressed as the mean OD obtained with the MAb 19D9D6 against 2.5 x 10^8 phages - the OD obtained with the anti borrelia burgdorferi OSPA MAb against the same number of phages.

Clones N°1-6 were then tested in parallel with a pool of 10 anti HCV core positive sera and a pool of 10 sera from healthy individuals :

Table 8

N° of clone	Clone sequence	OD (sera Core +) - OD (sera Core-)
1	WPHNWWPHFKVK SEQ ID NO:8	0.1
2	HLNILSTLWKYR SEQ ID NO:9	0.350
3	HKHAHNYRLPFS SEQ ID NO:10	0.286
4	PKVLIVVLEASG SEQ ID NO:11	0.362
5	QVYAEFKTSFRS SEQ ID NO:12	0
6	GHIHSMRHHRPT SEQ ID NO:13	0.310

The immunoreactivity of phage clones was tested according to the manufacturer manual. The results were expressed as the mean OD obtained with the pool of anti core positive human sera against 2.5 x 10^8 phages - the OD obtained with the pool of anti core negative human sera against the same number of phages. As shown in table 8, compared to the response obtained with negative sera, 5 out of the 6 tested clones gave a positive signal with the pool of positive sera. This results are in agreement with previous results, indicating that the epitope recognized by MAb 19D9D6 was also recognized by human sera.

CLAIMS

1. Polypeptide that specifically binds to antibodies that specifically bind to human hepatitis C virus consisting of an antigenic sequence having specific immunoreactivity to the antibodies of SEQ ID NO:1, with the proviso that said polypeptide is different from SEQ ID NO :1, SEQ ID NO :2 and sequences resulting from the amputation of SEQ ID NO :1 with 1 to 10 amino acids from the N-terminal part and/or the C-terminal part of SEQ ID NO :1.

2. The polypeptide according to claim 1, wherein said antigenic sequence consists of SEQ ID NO:1 modified with at least one modification selected from the group consisting of :

(1) substituting an amino acid of SEQ ID NO:1 for at least one homologous amino acid,

(2) a chemical modification.

3. The polypeptide according to claim 2, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophane and methionine.

4. The polypeptide according to claim 2, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from serine, threonine, tyrosine, asparagine, glutamine, cysteine, and glycine.

5. The polypeptide according to claim 2, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from aspartic acid and glutamic acid.

6. The polypeptide according to claim 2, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from lysine, arginine, histidine.

7. The polypeptide according to claim 2, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from glutamine and glutamic acid.

8. The polypeptide according to claim 2, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from asparagine and aspartic acid.

9. The polypeptide according to claim 2, wherein said chemical modification is at least one member selected from the group consisting of :

(i) replacement of an amino acid of the L series by an amino acid of the D series,

(ii) modification of side chains of amino acids,

(iii) modification of peptide bonds.

10. The polypeptide according to claim 9, wherein said modification of side chains is acetylation of amine functional groups, carboxymethylation of thiol functional groups or esterification of carboxylic functional groups.

11. The polypeptide according to claim 9, wherein said modification of peptide bonds is forming carba, retro-inverso, reduced and methylene-oxy bonds.

12. The polypeptide according to claim 1, being shorter than SEQ ID NO:1, and sharing at least one identical amino acid with SEQ ID NO:5.

13. The polypeptide according to claim 12, sharing at least two identical amino acids with SEQ ID NO:5.

14. The polypeptide according to claim 13, sharing at least two identical amino acids with SEQ ID NO:6 or at least one identical amino acid with SEQ ID NO:7.

15. The polypeptide according to claim 12, further having at least one amino acid which is homologous to at least one amino acid of SEQ ID NO: 1.

16. The polypeptide according to claim 15, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from alanine, leucine,

isoleucine, valine, proline, phenylalanine, tryptophane and methionine.

17. The polypeptide according to claim 15, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from serine, threonine, tyrosine, asparagine, glutamine, cysteine, and glycine.

18. The polypeptide according to claim 15, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from aspartic acid and glutamic acid.

19. The polypeptide according to claim 15, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from asparagine and aspartic acid.

20. The polypeptide according to claim 15, wherein said amino acid of SEQ ID NO:1 and said homologous amino acid are different and are selected from glutamine and glutamic acid.

21. The polypeptide according to claim 12, further having at least one amino acid which differs from at least one amino acid of SEQ ID NO:1 by a chemical modification of the side chain of said amino acid.

22. The polypeptide according to claim 21, wherein the chemical modification is selected from acetylation of an group, a carboxymethylation of a thiol group and an esterification of a carboxylic group.

23. The polypeptide according to claim 12, selected from the group consisting of SEQ ID NO:8 through SEQ ID NO:18.

24. A reagent for the detection of human hepatitis C virus, comprising as reactive substance at least one polypeptide according to claim 1.

FIG. 1

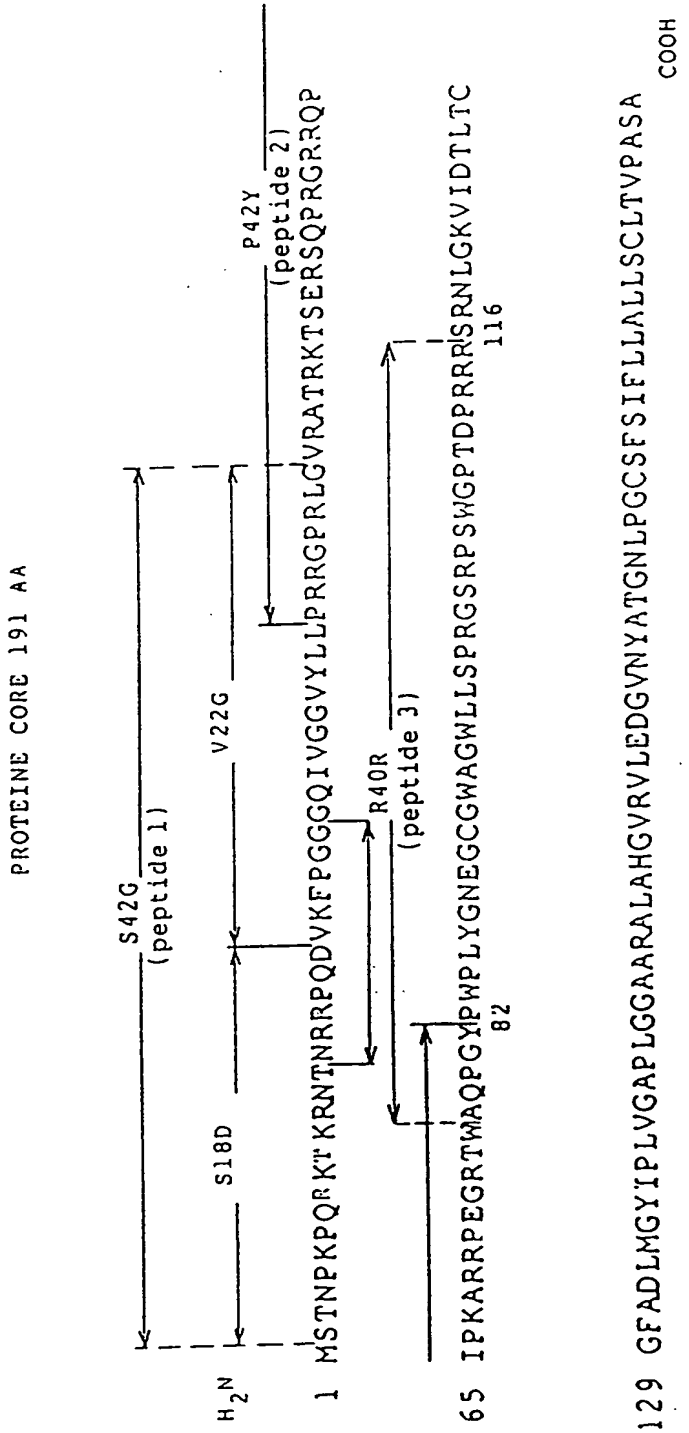


FIG. 2

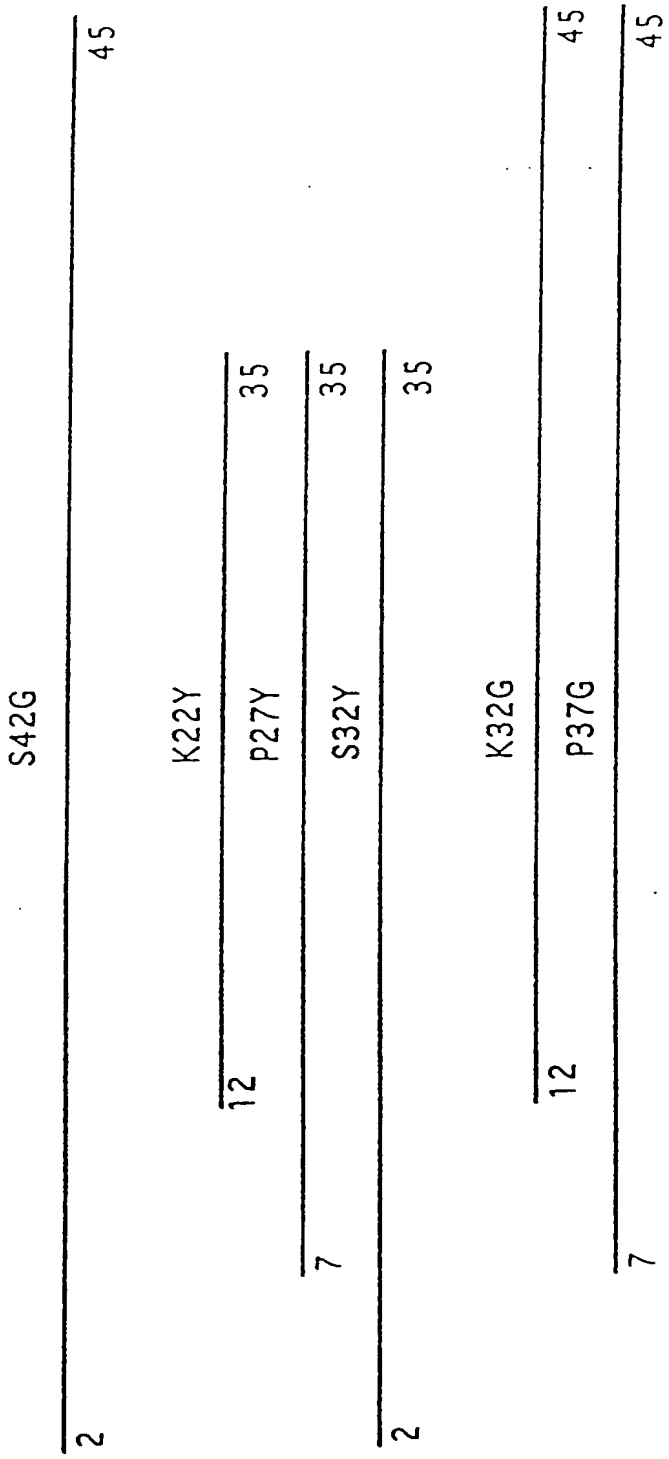


FIG. 3

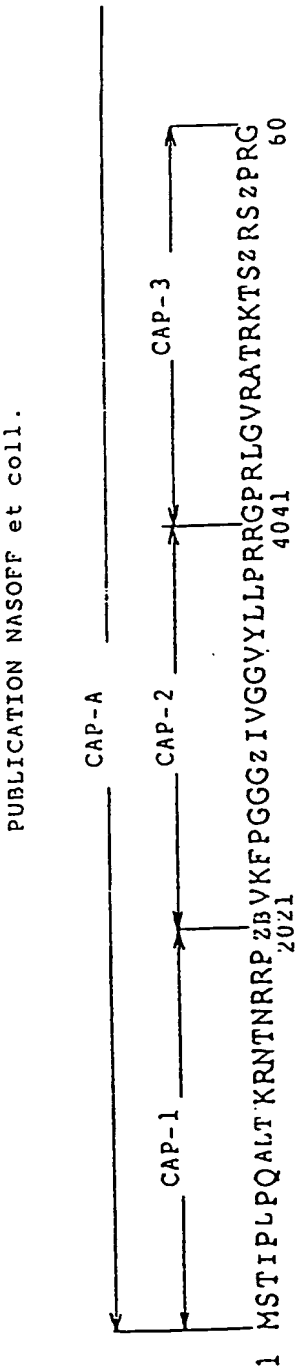


FIG. 4

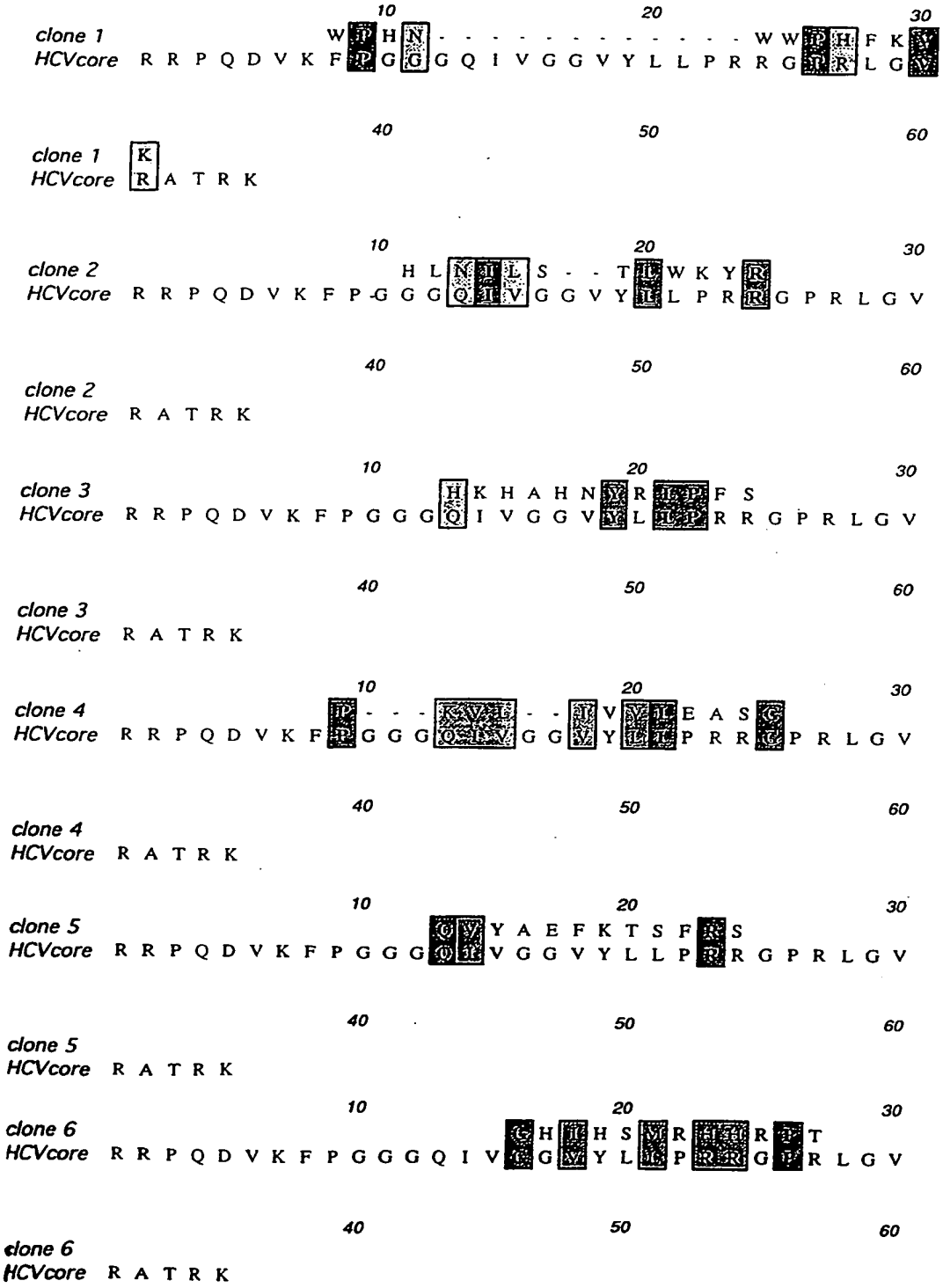
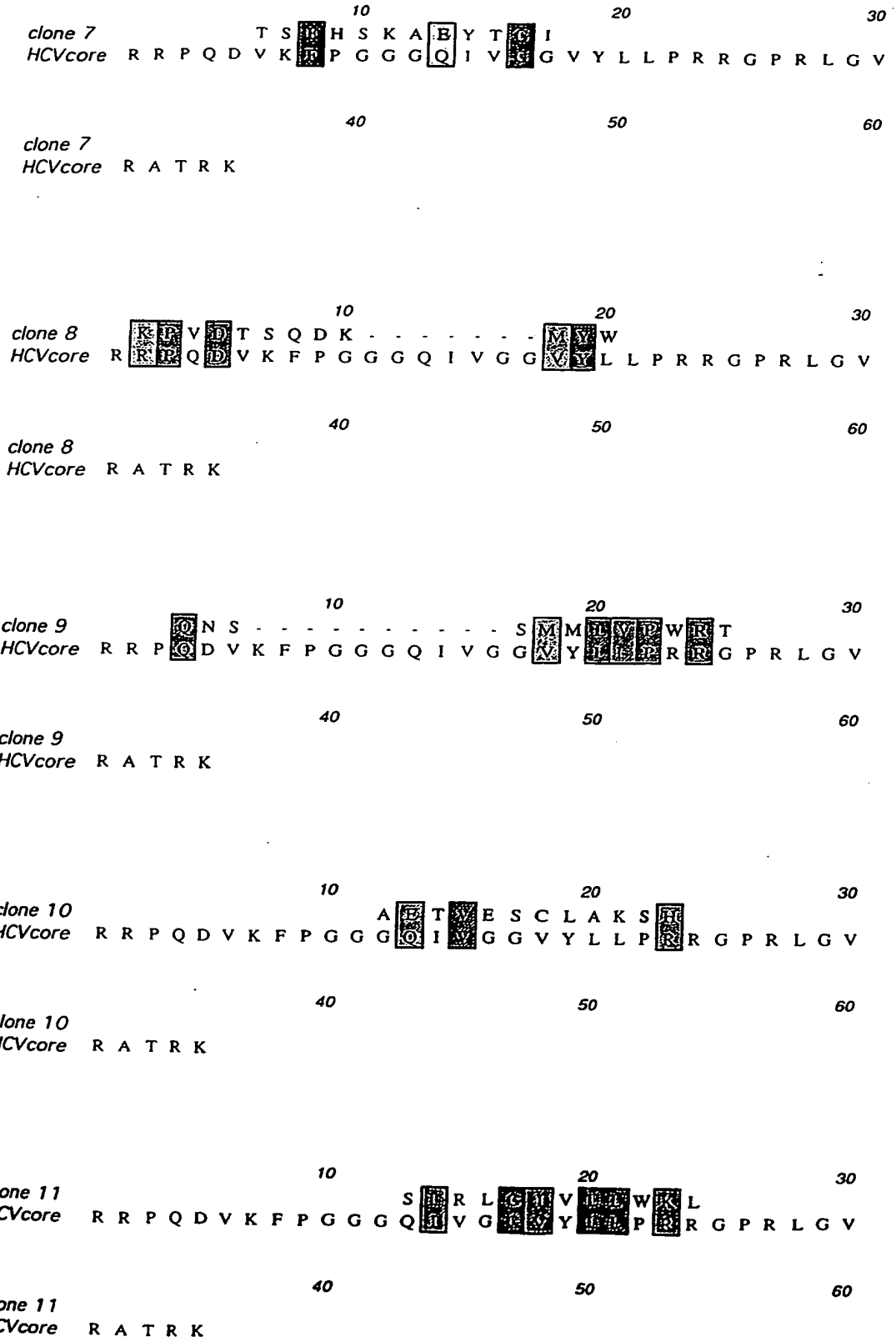


FIG. 4 (continued)



SEQUENCE LISTING

<110> BIO MERIEUX

<120> Synthetic polypeptides equivalent to polypeptides belonging to the hepatitis C virus (HCV) and applications

<130> Hépatite C 2

<140>
<141>

<150> US N°09/196,155
<151> 1998-11-20

<160> 18

<170> PatentIn Ver. 2.1

<210> 1
<211> 45
<212> PRT
<213> Homo sapiens

<400> 1
Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn
1 5 10 15

Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly
20 25 30

Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly
35 40 45

<210> 2
<211> 44
<212> PRT
<213> Homo sapiens

<400> 2
Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn Arg
1 5 10 15

Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly Gly
20 25 30

Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly
35 40

<210> 3
<211> 191
<212> PRT
<213> Homo sapiens

<400> 3
Met Ser Thr Asn Pro Lys Pro Gln Arg Lys Thr Lys Arg Asn Thr Asn
1 5 10 15

Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly
20 25 30

Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala
35 40 45

Thr Arg Lys Thr Ser Glu Arg Ser Gln Pro Arg Gly Arg Arg Gln Pro
50 55 60

Ile Pro Lys Ala Arg Arg Pro Glu Gly Arg Thr Trp Ala Gln Pro Gly
65 70 75 80

Tyr Pro Trp Pro Leu Tyr Gly Asn Glu Gly Cys Gly Trp Ala Gly Trp
85 90 95

Leu Leu Ser Pro Arg Gly Ser Arg Pro Ser Trp Gly Pro Thr Asp Pro
100 105 110

Arg Arg Arg Ser Arg Asn Leu Gly Lys Val Ile Asp Thr Leu Thr Cys
115 120 125

Gly Phe Ala Asp Leu Met Gly Tyr Ile Pro Leu Val Gly Ala Pro Leu
130 135 140

Gly Gly Ala Ala Arg Ala Leu Ala His Gly Val Arg Val Leu Glu Asp
145 150 155 160

Gly Val Asn Tyr Ala Thr Gly Asn Leu Pro Gly Cys Ser Phe Ser Ile
165 170 175

Phe Leu Leu Ala Leu Leu Ser Cys Leu Thr Val Pro Ala Ser Ala
180 185 190

<210> 4
<211> 60
<212> PRT
<213> Homo sapiens

<400> 4
Met Ser Thr Ile Pro Leu Pro Gln Ala Leu Thr Lys Arg Asn Thr Asn
1 5 10 15

Arg Arg Pro Glx Asx Val Lys Phe Pro Gly Gly Gly Glx Ile Val Gly
20 25 30

Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly Val Arg Ala
35 40 45

Thr Arg Lys Thr Ser Glx Arg Ser Glx Pro Arg Gly
50 55 60

<210> 5
<211> 29
<212> PRT
<213> Homo sapiens

<400> 5
Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln Ile Val Gly
1 5 10 15

Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly
20 25

<210> 6
<211> 16
<212> PRT
<213> Homo sapiens

<400> 6
Ile Val Gly Gly Val Tyr Leu Leu Pro Arg Arg Gly Pro Arg Leu Gly
1 5 10 15

<210> 7
<211> 13
<212> PRT
<213> Homo sapiens

<400> 7
Arg Arg Pro Gln Asp Val Lys Phe Pro Gly Gly Gly Gln
1 5 10

<210> 8
<211> 12
<212> PRT
<213> Homo sapiens

<400> 8
Trp Pro His Asn Trp Trp Pro His Phe Lys Val Lys
1 5 10

<210> 9
<211> 12
<212> PRT
<213> Homo sapiens

<400> 9
His Leu Asn Ile Leu Ser Thr Leu Trp Lys Tyr Arg
1 5 10

<210> 10
<211> 12
<212> PRT
<213> Homo sapiens

<400> 10
His Leu Asn Ile Leu Ser Thr Leu Trp Lys Tyr Arg
1 5 10

<210> 11
<211> 12
<212> PRT
<213> Homo sapiens

<400> 11
His Leu Asn Ile Leu Ser Thr Leu Trp Lys Tyr Arg
1 5 10

<210> 12
<211> 12
<212> PRT
<213> Homo sapiens

<400> 12
Gln Val Tyr Ala Glu Phe Lys Thr Ser Phe Arg Ser
1 5 10

<210> 13
<211> 12
<212> PRT
<213> Homo sapiens

<400> 13
Gly His Ile His Ser Met Arg His His Arg Pro Thr
1 5 10

<210> 14
<211> 12
<212> PRT
<213> Homo sapiens

<400> 14
Thr Ser Phe His Ser Lys Ala Glu Tyr Thr Gly Ile
1 5 10

<210> 15

<211> 12
<212> PRT
<213> Homo sapiens

<400> 15
Lys Asp Val Asp Thr Ser Gln Asp Lys Met Tyr Trp
1 5 10

<210> 16
<211> 12
<212> PRT
<213> Homo sapiens

<400> 16
Gln Asn Ser Ser Met Met Leu Val Pro Trp Arg Thr
1 5 10

<210> 17
<211> 12
<212> PRT
<213> Homo sapiens

<400> 17
Ala Glu Thr Val Glu Ser Cys Leu Ala Lys Ser His
1 5 10

<210> 18
<211> 12
<212> PRT
<213> Homo sapiens

<400> 18
Ser Leu Arg Leu Gly Ile Val Leu Leu Trp Lys Leu
1 5 10

INTERNATIONAL SEARCH REPORT

national Application No
PCT/IB 99/01933

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/18 G01N33/576

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 44 02 756 A (BOEHRINGER MANNHEIM GMBH) 3 August 1995 (1995-08-03) page 3, line 41; claims	1,2,12, 13,24
X	DE 44 30 998 A (BOEHRINGER MANNHEIM GMBH) 1 February 1996 (1996-02-01) the whole document	1,2,12, 13
X	WO 94 27153 A (CHIRON CORP) 24 November 1994 (1994-11-24) the whole document	1,2,12, 13
X	EP 0 518 313 A (MITSUBISHI CHEM IND) 16 December 1992 (1992-12-16) the whole document	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2000	28/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Chambonnet, F

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national Application No
PCT/IB 99/01933

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	JOLIVET-REYNAUD, C. ET AL.: "HCV core immunodominant region analysis using mouse monoclonal anibodies and human sera : characterization of major epitopes useful for antigen detection" JOURNAL OF MEDICAL VIROLOGY, vol. 56, no. 4, December 1998 (1998-12), pages 300-309, XP000904819 the whole document	1

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 99/01933

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4402756 A	03-08-1995	WO 9520764 A	03-08-1995
		EP 0741869 A	13-11-1996
		JP 9508374 T	26-08-1997
DE 4430998 A	01-02-1996	AT 187732 T	15-01-2000
		AU 682278 B	25-09-1997
		AU 3164995 A	22-02-1996
		AU 689626 B	02-04-1998
		AU 3165095 A	22-02-1996
		AU 690315 B	23-04-1998
		AU 3220595 A	22-02-1996
		CA 2195648 A	08-02-1996
		CA 2195752 A	08-02-1996
		CN 1134154 A,B	23-10-1996
		CN 1152923 A	25-06-1997
		DE 59507438 D	20-01-2000
		WO 9603650 A	08-02-1996
		WO 9603651 A	08-02-1996
		WO 9603409 A	08-02-1996
		WO 9603410 A	08-02-1996
		EP 0772616 A	14-05-1997
		EP 0774119 A	21-05-1997
		EP 0774120 A	21-05-1997
		EP 0720614 A	10-07-1996
		FI 961349 A	25-03-1996
		FI 970299 A	24-01-1997
		FI 970300 A	24-01-1997
		JP 9508473 T	26-08-1997
		JP 10506708 T	30-06-1998
		JP 2771900 B	02-07-1998
		JP 8509995 T	22-10-1996
		JP 10503485 T	31-03-1998
		KR 183420 B	15-05-1999
		NO 961161 A	21-03-1996
		NO 970130 A	10-01-1997
		NO 970197 A	16-01-1997
		NZ 290810 A	26-01-1998
		NZ 290811 A	19-12-1997
		NZ 291154 A	24-11-1997
		US 5958783 A	28-09-1999
		US 5981286 A	09-11-1999
		AU 688953 B	19-03-1998
		AU 3220495 A	22-02-1996
		AU 684992 B	08-01-1998
		AU 3220695 A	22-02-1996
		CA 2195753 A	08-02-1996
		CN 1130910 A	11-09-1996
		CN 1157655 A	20-08-1997
		DE 4430972 A	01-02-1996
		DE 4430973 A	01-02-1996
		DE 4439345 A	01-02-1996
		DE 4439346 A	01-02-1996
		DE 4439347 A	01-02-1996
WO 9427153 A	24-11-1994	CA 2162250 A	24-11-1994
		CN 1125982 A	03-07-1996
		CZ 9502929 A	15-05-1996
		EP 0698216 A	28-02-1996

INTERNATIONAL SEARCH REPORT

Information on patent family members

National Application No
PCT/IB 99/01933

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9427153 A		HU 73384 A	29-07-1996
		JP 8510329 T	29-10-1996
		PL 311653 A	04-03-1996
		PL 175360 B	31-12-1998
		PL 175338 B	31-12-1998
		SK 135995 A	04-09-1996
EP 0518313 A	16-12-1992	CA 2070952 A	12-12-1992
		JP 6000085 A	11-01-1994
		JP 6000086 A	11-01-1994

Form PCT/ISA/210 (patent family annex) (July 1992)